



DEPARTMENT OF AGRICULTURE

Animal and Plant Health Inspection Service

[Docket No. APHIS-2023-0022]

Movement of Organisms Modified or Produced through Genetic Engineering; Notice of Proposed Exemptions

AGENCY: Animal and Plant Health Inspection Service, USDA.

ACTION: Notice.

SUMMARY: We are advising the public that we are proposing to add five new types of genetic modifications a plant can contain and be exempt from the regulations for the movement of organisms modified or produced through genetic engineering because such modifications could otherwise be achieved through conventional breeding methods. First, we propose any diploid or autopolyploid plant with any combination of loss of function modifications (i.e., a modification that eliminates a gene's function) in one to all alleles of a single genetic locus, or any allopolyploid plant with any combination of loss of function modifications in one or both alleles of a single genetic locus on up to four pairs of homoeologous chromosomes, without the insertion of exogenous DNA, would qualify for exemption. Second, we propose that any diploid or autopolyploid plant in which the genetic modification is a single contiguous deletion of any size, resulting from cellular repair of one or two targeted DNA breaks on a single chromosome or at the same location(s) on two or more homologous chromosomes, without insertion of DNA, or with insertion of DNA in the absence of a repair template, would qualify for exemption. Third, we propose to extend the modifications described in certain existing exemptions in the regulations to all alleles of a genetic locus on the homologous chromosomes of an autopolyploid plant. Fourth, we propose that plants with up to four modifications that individually qualify for exemption and are made simultaneously or sequentially would be exempt from regulation, provided that each modification is at a different genetic locus. Fifth, we propose that plants that

have previously completed a voluntary review confirming exempt status and that have subsequently been produced, grown, and observed consistent with conventional breeding methods appropriate for the plant species, could be successively modified in accordance with the exemptions. This action would reduce the regulatory burden for developers of certain plants modified using genetic engineering that are not expected to pose plant pest risks greater than the plant pest risks posed by plants modified by conventional breeding methods.

DATES: We will consider all comments that we receive on or before [INSERT DATE 30 DAYS AFTER DATE OF PUBLICATION IN THE FEDERAL REGISTER].

ADDRESSES: You may submit comments by either of the following methods:

- Federal eRulemaking Portal: Go to <http://www.regulations.gov>. Enter APHIS-2023-0022 in the Search field. Select the Documents tab, then select the Comment button in the list of documents.
- Postal Mail/Commercial Delivery: Send your comment to Docket No. APHIS-2023-0022, Regulatory Analysis and Development, PPD, APHIS, Station 3A-03.8, 4700 River Road Unit 118, Riverdale, MD 20737-1238.

Supporting documents and any comments we receive on this docket may be viewed at [regulations.gov](http://www.regulations.gov) or in our reading room, which is located in room 1620 of the USDA South Building, 14th Street and Independence Avenue SW, Washington, DC. Normal reading room hours are 8 a.m. to 4:30 p.m., Monday through Friday, except holidays. To be sure someone is there to help you, please call (202) 799-7039 before coming.

FOR FURTHER INFORMATION CONTACT: Dr. Neil Hoffman, Science Advisor, Biotechnology Regulatory Services, APHIS, 4700 River Road Unit 98, Riverdale, MD 20737-1238; Neil.E.Hoffman@usda.gov; (301) 851-3947.

SUPPLEMENTARY INFORMATION:

The regulations in 7 CFR part 340 govern the movement (importation, interstate movement, or release into the environment) of certain organisms modified or produced through

genetic engineering. The Animal and Plant Health Inspection Service (APHIS) first issued these regulations in 1987 under the authority of the Federal Plant Pest Act of 1957 and the Plant Quarantine Act of 1912, two acts that were subsumed into the Plant Protection Act (PPA, 7 U.S.C. 7701 *et seq.*) in 2000, along with other provisions. Since 1987, APHIS has amended the regulations seven times, in 1988, 1990, 1993, 1994, 1997, 2005, and 2020.

On May 18, 2020, we published in the *Federal Register* (85 FR 29790-29838, Docket No. APHIS-2018-0034) a final rule¹ that marked the first comprehensive revision of the regulations since they were established in 1987. The final rule provided a clear, predictable, and efficient regulatory pathway for innovators, facilitating the development of organisms developed using genetic engineering that are unlikely to pose plant pest risks.

The May 2020 final rule included regulatory exemptions for certain categories of modified plants. Specifically, § 340.1(b) exempted plants that contain a single modification of one of the following types, specified in § 340.1(b)(1) through (3):

- The genetic modification is a change resulting from cellular repair of a targeted DNA break in the absence of an externally provided repair template; or
- The genetic modification is a targeted single base pair substitution; or
- The genetic modification introduces a gene known to occur in the plant's gene pool or makes changes in a targeted sequence to correspond to a known allele of such a gene or to a known structural variation present in the gene pool.

In addition to the modifications listed above, § 340.1(b)(4) provides that the Administrator may propose to exempt plants with additional modifications, based on what could be achieved through conventional breeding. Such proposals may either be APHIS-initiated or may be initiated via a request that is accompanied by adequate supporting information and

¹ To view the final rule and supporting documents, go to <https://www.regulations.gov/docket/APHIS-2018-0034>.

submitted by another party. In either case, APHIS will publish a notice in the *Federal Register* of the proposal, along with the supporting documentation, and will request public comments. After reviewing the comments, APHIS will publish a subsequent notice in the *Federal Register* announcing its final determination. A list specifying modifications a plant can contain and be exempt pursuant to paragraph (b)(4) is available on the APHIS website at <https://www.aphis.usda.gov/aphis/ourfocus/biotechnology>.

On July 19, 2021, we published a notice in the *Federal Register* (86 FR 37988-37989, Docket No. APHIS-2020-0072) proposing to exempt plants with any of the following additional modifications:

- Cellular repair of a targeted DNA break in the same location on two homologous chromosomes, in the absence of a repair template, resulting in homozygous or heterozygous biallelic mutations, each of which is a loss of function mutation;
- Contiguous deletion of any size resulting from cellular repair of a targeted DNA break in the presence of an externally supplied repair template; or
- Cellular repair of two targeted DNA breaks on a single chromosome or at the same location on two homologous chromosomes, when the repair results in a contiguous deletion of any size in the presence or absence of a repair template, or in a contiguous deletion of any size combined with an insertion of DNA in the absence of a repair template.

We received comments on that notice that suggested these exemptions were piecemeal and could be replaced with an overarching exemption. Furthermore, comments included additional exemptions beyond those that we proposed.

Based on the comments that we received and our own subsequent review and analysis of conventional breeding techniques that are currently employed, we are withdrawing the original three proposed exemptions and are proposing five new types of modifications a plant can contain and qualify for exemption from regulation pursuant to paragraph (b)(4) of § 340.1.

First, we propose that a diploid or autopolyploid plant with any combination of loss of function modifications in one to all alleles of a single genetic locus, or an allopolyploid plant with any combination of loss of function modifications in one or both alleles of a single genetic locus on up to four pairs of homoeologous chromosomes, without the insertion of exogenous DNA, would qualify for exemption (proposed exemption 340.1(b)(4)(vi)(Additional Modification (AM)1)). (Because this exemption would be found solely on the internet, and not in the regulations themselves, the “AM” nomenclature would be used to identify the method by which it and the other exemptions proposed in this notice were added.) This category would apply to scenarios that might not be expressly described in the exemptions codified in the May 2020 final rule (namely, paragraphs (b)(1) and (2) of § 340.1) but would achieve an end result that can also be accomplished by those exemptions. In addition, it more broadly extends, compared to the 2020 rule, loss of function mutations without the insertion of exogenous DNA to polyploid plants.

Second, we propose that any diploid or autopolyploid plant in which the genetic modification is a single contiguous deletion of any size, resulting from cellular repair of one or two targeted DNA breaks on a single chromosome or at the same location(s) on two or more homologous chromosomes, without insertion of DNA, or with insertion of DNA in the absence of a repair template, would qualify for exemption (proposed exemption 340.1(b)(4)(vi)(AM2)). As proposed, additional modifications to homoeologous loci of homoeologous chromosomes of allopolyploids would not qualify for this exemption.

Third, we propose to extend the modifications described in the exemptions found at § 340.1(b)(2) and (3) to all alleles of a genetic locus on the homologous chromosomes of autopolyploids (proposed exemption 340.1(b)(4)(vi)(AM3)). As proposed, additional modifications to homoeologous loci of homoeologous chromosomes of allopolyploids would not qualify for this exemption.

Fourth, we propose that plants with up to four modifications of a certain type, made simultaneously or sequentially, that individually qualify for exemption, and provided each modification is at a different genetic locus, would be exempt from regulation because such modifications are achievable through conventional breeding methods (proposed exemption 340.1(b)(4)(vi)(AM4)). Allopolyploid plants could contain up to four of the proposed loss of function modifications described herein or four modifications described under § 340.1(b)(2) and (3) or a combination thereof, provided each modification is introduced into just one allele; however, allopolyploid plants would not be exempt if they contain a modification that is allowable only in diploid and autopolyploid plants.

Fifth, we propose that plants that have previously completed voluntary reviews confirming the plants' exempt status as described in § 340.1(e), which provides the process by which developers can request such a confirmation of exempt status, and that have been produced, grown, and observed consistent with conventional breeding methods appropriate for the plant species, could be successively modified in accordance with any exemption under § 340.1(b) of the regulations (proposed exemption 340.1(b)(4)(vi)(AM5)).

We are also making available for public review scientific literature that we considered prior to initiating this notice, which demonstrates that in polyploid plants (such as wheat, potato, tobacco, and canola), all alleles of a single genetic locus can be modified by conventional breeding to generate loss of function mutations. This notice provides scientific literature supporting our rationale for why the proposed modifications could extend to any autopolyploid species and our rationale for why some of the proposed modifications could extend to any allopolyploid species. This notice includes examples of conventional breeding programs in sterile crops such as banana, long cycle crops such as forest trees, crops with complex genomes such as strawberry and sugarcane, and highly heterozygous crops such as potato and apple. This notice discusses literature describing the approach of pyramiding genes (i.e., the simultaneous selection for and/or introduction of multiple genes during plant breeding) to create multiplex

edits and provide examples in soybean, coffee, tobacco, tomato, potato, corn, and rice where four to seven traits are pyramided by conventional breeding methods. We also provide references to literature describing how homozygous autopolyploids can be created through conventional breeding methods in autopolyploid plants that are not applicable to allopolyploids plants. We also explain how the categories for loss of function modifications, and successive modifications for plants that have completed the voluntary confirmation process and that have been produced, grown, and observed are consistent with conventional breeding methods for the appropriate plant species. This action would reduce the regulatory burden for developers of certain plants modified using genetic engineering that are not expected to pose plant pest risks greater than the plant pest risks posed by plants modified by conventional breeding methods and, thus, should not be subjected to regulation under part 340.

First Proposed Exemption

Commenters to the previous July 2021 notice suggested that we “establish a single exemption category for indel modifications resulting from modifications to the alleles of a single gene on homologous chromosomes.” We recognize that as new tools emerge, there may be DNA modifications that are not expressly covered by the three exemptions described in the July 2021 notice. For example, base editing and prime editing involve nicking a single strand rather than making double strand breaks. In the case of base editing, a deaminase further modifies the DNA before the changes are resolved by natural repair. In prime editing, prime-editing guide RNA contains an internal template and further uses reverse transcriptase to incorporate the edit. When base editing is used to introduce a loss of function (“LOF”) mutation to a single genetic locus, multiple changes may occur within the single genetic locus. The fact that multiple changes occur is irrelevant if one or more of the changes leads to a loss of function. Both base-editing and prime-editing can be used to make modifications that conform to the spirit of the modifications codified in § 340.1(b)(1) that are exempt from regulation, but they are not expressly described in the modifications. Creating a category for any DNA modification that

leads to LOF of a single gene on homologous chromosomes would cover scenarios we did not specifically describe that are nevertheless consistent with our intent for modifications that would qualify for exemption in § 340.1(b)(1) because they are achievable through conventional breeding methods.

Accordingly, in this notice, we propose that diploid or autopolyploid plants with any combination of loss of function modifications in one to all alleles of a single genetic locus, or allopolyploid plants with any combination of loss of function modifications in one or both alleles of a single genetic locus on up to four homoeologous chromosomes, without the insertion of exogenous DNA, would be exempt from regulation. In the comment period for the previous notice, several papers were brought to our attention describing the successful breeding of tetraploid (AABB genomes) and hexaploid (AABBDD genomes) wheat lines with loss of function alleles for all four or six homoeologous alleles, respectively. In one case,² homologous null mutations in starch synthase from both the A and B genomes were isolated from the M2 generation of ethyl methanesulfonate (EMS) mutagenized tetraploid wheat lines. Both null mutants were crossed to generate the null lacking all 4-functioning starch synthase alleles. In a second case,³ the exomes of 2735 EMS mutagenized lines were sequenced, and more than 10 million mutations were identified covering about 90 percent of the three wheat genomes. The authors explained how loss of function homozygous mutants could be successfully isolated from both genomes in the third generation of tetraploid wheat and homozygous mutants across all three genomes in the fourth generation of a hexaploid wheat. The literature contains several additional cases of double and triple null mutants successfully created by conventional breeding

² Li, S., X. Zhong, X. Zhang, M. M. Rahman, J. Lan, H. Tang, P. Qi, J. Ma, J. Wang, G. Chen, X. Lan, M. Deng, Z. Li, W. Harwood, Z. Lu, Y. Wei, Y. Zheng and Q. Jiang (2020). "Production of waxy tetraploid wheat (*Triticum turgidum durum* L.) by EMS mutagenesis." *Genetic Resources and Crop Evolution* 67(2): 433-443).

³ Krasileva, K. V., H. A. Vasquez-Gross, T. Howell, P. Bailey, F. Paraiso, L. Clissold, J. Simmonds, R. H. Ramirez-Gonzalez, X. Wang, P. Borrill, C. Fosker, S. Ayling, A. L. Phillips, C. Uauy and J. Dubcovsky (2017). "Uncovering hidden variation in polyploid wheat." *Proc Natl Acad Sci U S A* 114(6): E913-e921).

(naturally occurring transposon induced mutation/ems mutagenesis, tilling, and marker assisted breeding) in the polyploids, wheat, tobacco, potato and canola.⁴ The combination of mutagenesis and exome-sequencing described by Krasileva et al. 2017, has also been applied in tetraploid tobacco.⁵ Based on these examples, it appears this methodology can be used to create the modifications captured by the exemption in any species that can be bred conventionally. Breeding programs exist for crops that are challenging to breed, such as the largely sterile triploid bananas,⁶ forest trees with long generation times,⁷ and crops with complex genomes such

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- ⁴ Pearce, S., L. M. Shaw, H. Lin, J. D. Cotter, C. Li and J. Dubcovsky (2017). “Night-Break Experiments Shed Light on the Photoperiod1-Mediated Flowering” *Plant Physiology* 174(2): 1139-1150; Karunarathna, N. L., H. Wang, H.-J. Harloff, L. Jiang and C. Jung (2020). “Elevating seed oil content in a polyploid crop by induced mutations in SEED FATTY ACID REDUCER genes.” *Plant Biotechnology Journal* 18(11): 2251-2266; Kippes, N., Chen, A., Zhang, X., Lukaszewski, A.J., and Dubcovsky, J. (2016). Development and characterization of a spring hexaploid wheat line with no functional VRN2 genes. *Theor Appl Genet* 129, 1417-1428. Lewis, R.S., Lopez, H.O., Bowen, S.W., Andres, K.R., Steede, W.T., and Dewey, R.E. (2015). Transgenic and Mutation-Based Suppression of a Berberine Bridge Enzyme-Like (BBL) Gene Family Reduces Alkaloid Content in Field-Grown Tobacco. *PLOS ONE* 10, e0117273. Mccord, P., Zhang, L., and Brown, C. (2012). The Incidence and Effect on Total Tuber Carotenoids of a Recessive Zeaxanthin Epoxidase Allele (Zep1) in Yellow-fleshed Potatoes. *American Journal of Potato Research* 89, 262-268.
- ⁵ Udagawa, H., Ichida, H., Takeuchi, T., Abe, T., and Takakura, Y. (2021). Highly Efficient and Comprehensive Identification of Ethyl Methanesulfonate-Induced Mutations in *Nicotiana tabacum* L. by Whole-Genome and Whole-Exome Sequencing. *Front Plant Sci* 12, 671598.
- ⁶ Jenny, C., Tomekpe, K., Bakry, F., and Escalent, J.V. (2002). “Conventional Breeding of Bananas”, in: *Mycosphaerella leaf spot diseases of bananas: present status and outlook*. (eds.) L. Jacome, P. Lepoiver, D. Marin, R. Ortiz, R. Romero & J.V. Escalent. (San Jose Costa Rica: INIBAP).
- ⁷ Harfouche, A., Meilan, R., Kirst, M., Morgante, M., Boerjan, W., Sabatti, M., and Scarascia Mugnozza, G. (2012). Accelerating the domestication of forest trees in a changing world. *Trends in Plant Science* 17, 64-72.

as strawberry⁸ and sugarcane,⁹ or highly heterozygous genomes such as potato¹⁰ or apple.¹¹ We propose that any diploid or autopolyploid plant that contains any combination of loss of function modifications in one to all alleles of a single genetic locus without the insertion of exogenous DNA, or any allopolyploid plant with any combination of loss of function modifications in one or both alleles of a single genetic locus on up to four homoeologous chromosomes, would qualify for exemption because such modifications are achievable through conventional breeding methods. The limitation to four homoeologous chromosomes in polyploid plants is explained further below.

Modifications resulting from insertions of exogenous DNA do not currently qualify for exemption and, likewise, LOF mutations created through insertion of exogenous DNA such as T-DNA (the transferred DNA of the (Ti) plasmid of *Agrobacterium* used in the transformation of plant cells) or transposons (DNA sequences that can move and integrate to different locations within the genome), would not qualify for exemption as proposed. LOF mutations could qualify for more than one exemption. For example, LOF mutations may still qualify for exemption § 340.1(b)(3), if they are already known to occur in the gene pool of the plant species.

By loss of function, we mean a mutation in which the altered gene product prevents the normal gene product from being produced or renders it inactive.¹² By gain of function (GOF)

⁸ Hummer, K.E., and Hancock, J. (2009). Strawberry genomics: botanical history, cultivation, traditional breeding, and new technologies. *Genetics and genomics of Rosaceae*, 413-435.

⁹ Kumar, U., Priyanka, and Kumar, S. (2016). “Genetic Improvement of Sugarcane Through Conventional and Molecular Approaches”, 325-342.

¹⁰ Bonierbale, M.W., Amoroso, W.R., Salas, E., and De Jong, W. (2020). “Potato Breeding”, in *The Potato Crop: Its Agricultural, Nutritional and Social Contribution to Humankind*, eds. H. Campos & O. Ortiz. (Cham: Springer International Publishing), 163-217; Bethke, P.C., Halterman, D.A., Francis, D.M., Jiang, J., Douches, D.S., Charkowski, A.O., and Parsons, J. (2022). Diploid Potatoes as a Catalyst for Change in the Potato Industry. *American Journal of Potato Research* 99, 337-357.

¹¹ Sedov, E.N. (2014). Apple breeding programs and methods, their development and improvement. *Russian Journal of Genetics: Applied Research* 4, 43-51.

¹² King, R., Stansfield, W., & Mulligan, P. (2007). loss of function mutation. In *A Dictionary of Genetics*. Oxford University Press. Retrieved 6 Jun. 2023, from

mutation, we mean a mutation that alters the properties of the protein product so that it has novel properties or has greater activity because a regulatory site has been lost¹³ and is usually dominant, semidominant, or codominant. In some cases, a mutation can render a protein to be non-functioning but lead to a new phenotype. For example, mutations that knockout the repressor protein CLV3 (CLAVATA 3) result in larger sized fruit.¹⁴ These mutations are a LOF modification that would qualify for exemption. In cases where a deletion or frameshift mutation leads to a new molecular function or increased expression of the altered gene product, the modification would not qualify for the new exemption. For example, a codon deletion in protoporphyrinogen oxidase conferred resistance to PPO type herbicide inhibitors.¹⁵ This deletion results in a protein with a new molecular function, is dominant, and does not lack the molecular function of the wild type (it is still able to convert protoporphyrinogen IX to protoporphyrin IX). This particular example is a naturally occurring mutation described in *Amaranthus tuberculatus*. If genome editing were used to confer herbicide tolerance to a crop plant by deleting the corresponding codon by DNA break and repair, the modified plant would likely qualify for the exemption found at § 340.1(b)(1). Thus, although GOF mutations will not

<https://www.oxfordreference.com/view/10.1093/acref/9780195307610.001.0001/acref-9780195307610-e-3651>.

¹³ Lackie, J. gain-of-function mutation. In Nation, B. (Ed.), *A Dictionary of Biomedicine*.: Oxford University Press. Retrieved 6 Jun. 2023, from <https://www.oxfordreference.com/view/10.1093/acref/9780191829116.001.0001/acref-9780191829116-e-3735>.

¹⁴ Rodríguez-Leal, D., Lemmon, Z.H., Man, J., Bartlett, M.E., and Lippman, Z.B. (2017). Engineering Quantitative Trait Variation for Crop Improvement by Genome Editing. *Cell* 171, 470-480.e478.

Rönspies, M., Schindele, P., and Puchta, H. (2021). CRISPR/Cas-mediated chromosome engineering: opening up a new avenue for plant breeding. *J Exp Bot* 72, 177-183.

Xu, C., Liberatore, K.L., Macalister, C.A., Huang, Z., Chu, Y.-H., Jiang, K., Brooks, C., Ogawa-Ohnishi, M., Xiong, G., Pauly, M., Van Eck, J., Matsubayashi, Y., Van Der Knaap, E., and Lippman, Z.B. (2015). A cascade of arabinosyltransferases controls shoot meristem size in tomato. *Nature Genetics* 47, 784-792.

¹⁵ Patzoldt, W.L., Hager, A.G., McCormick, J.S., and Tranel, P.J. (2006). A codon deletion confers resistance to herbicides inhibiting protoporphyrinogen oxidase. *Proceedings of the National Academy of Sciences* 103, 12329-12334.

qualify for the proposed exemption 340.1(b)(4)(vi)(AM4) as listed in the above-mentioned exemptions-confirmations website, there are some GOF mutations that could meet the criteria for exemptions at § 340.1(b)(1) through (3). For example, promoter deletions can result in either LOF or GOF. If a promoter deletion eliminates or greatly decreases expression of the downstream gene, that would be a LOF modification and would qualify for this exemption or the § 340.1(b)(1) exemption. If the promoter deletion results in an increase of expression of the downstream gene, that would be a GOF modification and it would not qualify for this exemption but would qualify for the § 340.1(b)(1) exemption. In any plant, GOF modifications from faulty DNA repair qualify under exemptions § 340.1(b)(1) for a DNA break on a single chromosome or at the same location on two homologous chromosomes. In addition, GOF modifications from faulty repair could qualify for exemption under 340.1(b)(4)(vi) AM2 for one or two DNA breaks to the same location in the absence of an external template on all homologous chromosomes in autopolyploids (see below). In short, our proposal does not extend to all modifications that involve the insertion or deletion of bases (“indel”) because GOF modifications are statistically less common than LOF mutations and the same GOF mutation would not be expected to occur across multiple alleles in allopolyploids by conventional breeding.

We welcome comments from the public on the scope of this proposed exemption.

Second Proposed Exemption

In the published notice of July 2021, we proposed that plants with a modification that results in a single contiguous deletion of any size using an external repair template or using two targeted DNA breaks on a single chromosome would be exempt from regulation because they are achievable through conventional breeding methods. This type of modification allows deletions to contain more than one genetic locus. Based on the comments and information we received in response to the July 2021 notice, we are clarifying how the contiguous deletion of any size would apply to polyploids. Based on examples and methods described above, we propose that any diploid or autopolyploid plant with a genetic modification that is a single contiguous deletion

of any size, resulting from cellular repair of one or two targeted DNA breaks on a single chromosome or at the corresponding location(s) on two or more homologous chromosomes, without insertion of DNA, or with the insertion of DNA in the absence of a repair template, would be exempt because these modifications are achievable through conventional breeding methods. This proposed modification allows for multiple modifications in autopolyploids, but not allopolyploids. This is because the literature indicates this type of modification can be achieved through conventional breeding in autopolyploids to produce the same deletion throughout the genome. For example, though potato is highly heterozygous, a highly homozygous line was established from a doubled monoploid derived by another culture of a heterozygous diploid¹⁶ and this line in turn was used to create homozygous tetraploid lines by another round of whole genome doubling.¹⁷ In this way, conventional breeding was used to produce homozygous autopolyploids from allele variants in the haploid genome. Additionally, through random assortment of homologous chromosomes in autopolyploids, it is possible to achieve homozygosity of a modification across all chromosomes, while maintaining a high degree of heterozygosity across a genome, particularly when double reduction progeny are

¹⁶ Xu, X., Pan, S., Cheng, S., Zhang, B., Mu, D., Ni, P., Zhang, G., Yang, S., Li, R., Wang, J., Orjeda, G., Guzman, F., Torres, M., Lozano, R., Ponce, O., Martinez, D., De La Cruz, G., Chakrabarti, S.K., Patil, V.U., Skryabin, K.G., Kuznetsov, B.B., Ravin, N.V., Kolganova, T.V., Beletsky, A.V., Mardanov, A.V., Di Genova, A., Bolser, D.M., Martin, D.M.A., Li, G., Yang, Y., Kuang, H., Hu, Q., Xiong, X., Bishop, G.J., Sagredo, B., Mejía, N., Zagorski, W., Gromadka, R., Gawor, J., Szczesny, P., Huang, S., Zhang, Z., Liang, C., He, J., Li, Y., He, Y., Xu, J., Zhang, Y., Xie, B., Du, Y., Qu, D., Bonierbale, M., Ghislain, M., Del Rosario Herrera, M., Giuliano, G., Pietrella, M., Perrotta, G., Facella, P., O'brien, K., Feingold, S.E., Barreiro, L.E., Massa, G.A., Diambra, L., Whitty, B.R., Vaillancourt, B., Lin, H., Massa, A.N., Geoffroy, M., Lundback, S., Dellapenna, D., Robin Buell, C., Sharma, S.K., Marshall, D.F., Waugh, R., Bryan, G.J., Destefanis, M., Nagy, I., Milbourne, D., Thomson, S.J., Fiers, M., Jacobs, J.M.E., Nielsen, K.L., Sønderkær, M., Iovene, M., Torres, G.A., Jiang, J., Veilleux, R.E., Bachem, C.W.B., De Boer, J., Borm, T., Kloosterman, B., Van Eck, H., Datema, E., Te Lintel Hekkert, B., Goverse, A., Van Ham, R.C.H.J., Visser, R.G.F., The Potato Genome Sequencing, C., The Potato Genome, C., Shenzhen, B.G.I., et al. (2011). Genome sequence and analysis of the tuber crop potato. *Nature* 475, 189-195.

¹⁷ Guo, H., Zhou, M., Zhang, G., He, L., Yan, C., Wan, M., Hu, J., He, W., Zeng, D., Zhu, B., and Zeng, Z. (2023). Development of homozygous tetraploid potato and whole genome doubling-induced the enrichment of H3K27ac and potentially enhanced resistance to cold-induced sweetening in tubers. *Horticulture Research* 10.

selected.¹⁸ Based on our review of the literature, we believe that this type of modification is not possible through conventional breeding methods for allopolyploids, which is why the proposed modification applies only to autopolyploids.

Third Proposed Exemption

We propose to extend the modifications described in § 340.1(b)(2) and (3) to all alleles of a genetic locus on the homologous chromosomes of autopolyploids. This would allow the following modifications to all alleles of a single gene on all homologous chromosomes in autopolyploids:

- a targeted single base pair substitution, or
- introduction of a gene known to occur in the plant's gene pool or make changes in a targeted sequence to correspond to a known allele of such a gene or to a known structural variation present in the gene pool.

For the reasons discussed above, the modifications described in § 340.1(b)(2) and (3) would only extend to all loci on the homologous chromosomes in autopolyploids plants and not to all homoeologous loci in allopolyploids plants.

Fourth Proposed Exemption

We have received several comments that multiplexing genome edits that individually qualify for exemption should qualify for exemption when achieved simultaneously or sequentially because conventional breeding allows the combination of multiple desired traits. In the 2020 preamble, APHIS noted, “[i]nitially, the exemptions will apply only to plants containing a single targeted modification in one of the categories listed. APHIS anticipates scientific information and/or experience may, over time, allow APHIS to list additional modifications that plants can contain and still be exempted from the regulations so that the

¹⁸ Bourke, P.M., Voorrips, R.E., Visser, R.G., and Maliepaard, C. (2015). The Double-Reduction Landscape in Tetraploid Potato as Revealed by a High-Density Linkage Map. *Genetics* 201, 853-863.

regulatory system stays up to date and keeps pace with advances in scientific knowledge, evidence, and experience. This may include multiple simultaneous genomic changes.” 85 Fed. Reg. 29790, 29794. We have verified that there is literature on this topic, including literature describing gene pyramiding.¹⁹ One commenter provided us with a patent for a tobacco plant made homozygous in five separate loci through conventional breeding.²⁰ Additionally, we observed cases where four to seven traits were combined in soybean,²¹ potato,²² coffee,²³ corn,²⁴ tomato,²⁵ and rice²⁶ suggesting that pyramiding genes is becoming a standard practice in conventional breeding and four traits are conservatively within the norm. The examples

¹⁹ Majhi, P. (2020). "GENE PYRAMIDING.", 3-16; Chapagain, S., Pruthi, R., and Subudhi, P.K. (2023). Pyramiding QTLs using multiparental advanced generation introgression lines enhances salinity tolerance in rice. *Acta Physiologiae Plantarum* 45, 59.; Dormatey, R., Sun, C., Ali, K., Coulter, J.A., Bi, Z., and Bai, J. (2020). Gene Pyramiding for Sustainable Crop Improvement against Biotic and Abiotic Stresses. *Agronomy* 10, 1255.; Malav, A.K., Indu, and Chandrawat, K.S. (2016). Gene Pyramiding: An Overview. *International Journal of Current Research in Biosciences and Plant Biology* 3, 22-28; Muthurajan, R., and Balasubramanian, P. (2009). "Pyramiding Genes for Enhancing Tolerance to Abiotic and Biotic Stresses," in *Molecular Techniques in Crop Improvement: 2nd Edition*, eds. S.M. Jain & D.S. Brar. (Dordrecht: Springer Netherlands), 163-184; Servin, B., Martin, O., Mezard, M., and Hospital, F. (2004). Toward a Theory of Marker-Assisted Gene Pyramiding. *Genetics* 168, 513-523.

²⁰ Lewis, R.S., Dewey, R.E., and Tamburrino, J.S. (2023). US Patent Application for GENETIC APPROACH FOR ACHIEVING ULTRA LOW NICOTINE CONTENT IN TOBACCO Patent Application (Application #20230029171 issued January 26, 2023) - Justia Patents Search.

²¹ Singh, Y., Shrivastava, M., and Banerjee, J. (2021). "Chapter -3 Gene Pyramiding in Soybean."

²² Rogozina, E.V., Beketova, M.P., Muratova, O.A., Kuznetsova, M.A., and Khavkin, E.E. (2021). Stacking Resistance Genes in Multiparental Interspecific Potato Hybrids to Anticipate Late Blight Outbreaks. *Agronomy* 11, 115.

²³ Saavedra, L.M., Caixeta, E.T., Barka, G.D., Borém, A., Zambolim, L., Nascimento, M., Cruz, C.D., Oliveira, A.C.B.D., and Pereira, A.A. (2023). Marker-Assisted Recurrent Selection for Pyramiding Leaf Rust and Coffee Berry Disease Resistance Alleles in *Coffea arabica* L. *Genes* 14, 189.

²⁴ Zambrano, J.L., Jones, M.W., Brenner, E., Francis, D.M., Tomas, A., and Redinbaugh, M.G. (2014). Genetic analysis of resistance to six virus diseases in a multiple virus-resistant maize inbred line. *Theoretical and Applied Genetics* 127, 867-880.

²⁵ Hanson, P., Lu, S.-F., Wang, J.-F., Chen, W., Kenyon, L., Tan, C.-W., Tee, K.L., Wang, Y.-Y., Hsu, Y.-C., Schafleitner, R., Ledesma, D., and Yang, R.-Y. (2016). Conventional and molecular marker-assisted selection and pyramiding of genes for multiple disease resistance in tomato. *Scientia Horticulturae* 201, 346-354.

²⁶ Ramalingam, J., Raveendra, C., Savitha, P., Vidya, V., Chaithra, T.L., Velprabakaran, S., Saraswathi, R., Ramanathan, A., Arumugam Pillai, M.P., Arumugachamy, S., and Vanniarajan, C. (2020). Gene Pyramiding for Achieving Enhanced Resistance to Bacterial Blight, Blast, and Sheath Blight Diseases in Rice. *Frontiers in Plant Science* 11.

provided include four different diploid species, an autopolyploid species (potato), an allopolyploid species (coffee), which is also a tree, suggesting that gene pyramiding is widely applicable to crop plants. When discussing the first proposed exemption, we noted new techniques that created DNA modifications using chemical mutagenesis while characterizing the genome using molecular analysis both of which are applicable to any species. We also provide examples of crops that have active breeding programs even though they are challenging to breed. Based on feedback during the comment period of the 2021 notice and our own review of the literature, it is our current view that a single targeted modification is more conservative than what can be achieved by conventional breeding in all species.

Accordingly, we propose that plants with up to four modifications of a certain type that individually qualify for exemption and that are made simultaneously or sequentially would be exempt from regulation, provided that each modification is at a different genetic locus. This is because such modifications are achievable through conventional breeding methods. For the reasons discussed above, allopolyploid plants could contain up to four of the proposed loss of function modifications described herein. Allopolyploid plants would also qualify for exemption with the following changes to a single pair of homologous chromosomes:

- § 340.1(b)(2)-a genetic modification is a targeted single base pair substitution; and
- § 340.1(b)(3)-the introduction of a gene known to occur in the plant's gene pool or makes changes in a targeted sequence to correspond to a known allele of such a gene or to a known structural variation present in the gene pool.

We propose that up to four such modifications would qualify for exemption in allopolyploids provided that each change is heterozygous. We note that the introduction of multiple dominant resistance traits has been accomplished by conventional breeding in both allopolyploid coffee (see footnote 23) and autopolyploid potato (see footnote 22). However, we are not aware of multiple homologous traits pyramided in allopolyploids.

Modifications would be counted based on loci modified. For an autopolyploid, such as potato, which has four alleles of the same genetic locus, a change to make four homozygous copies of an allele would count as one multiplex modification. However, in an allopolyploid, such as canola, which has two pairs of homoeologous chromosomes, LOF edits to all alleles (two loci and four alleles) would count as two multiplex modifications. We welcome comments from the public on the number of individual modifications that are achievable simultaneously or sequentially in plants based on conventional breeding methods, and comments on the reasons for or against allowing for simultaneous or sequential modifications in all plants. We emphasize that multiplexed or sequential modifications must be made to distinct loci; multiple modifications to a single gene would not qualify for exemption except in the cases where the gene is known to occur in the plant's gene pool.

Fifth Proposed Exemption

We have also received questions on whether a modified plant that meets the criteria for exemption from the regulations at part 340, may undergo successive or further modification. In the preamble that accompanied the final rule, we noted that we would address the possibility for sequential modification (i.e., subsequent or further modification to an exempt plant) in a future notice using the process described in § 340.1(b)(4). In conventional breeding, it is standard practice to introduce new traits through successive crosses. Conventional breeding affords the opportunity to evaluate and select the progeny of a cross that will be advanced in the breeding program. Along these lines, we propose that plants that have previously completed the voluntary confirmation process (also called the “CR” process) found at § 340.1(e) and that have been produced, grown, and observed consistent with conventional breeding methods for the appropriate plant species, may be successively modified in accordance with the exemptions because allowing for such successive modification is consistent with plant development in conventional breeding programs. Plants that are merely hypothetical in nature would not be eligible for subsequent hypothetical modifications because they have not yet been produced,

grown, and observed consistent with conventional breeding methods for the appropriate plant species.

The following table summarizes the proposed exemptions and their applicability to polyploids:

Table 1. Summary of Proposed Exemption Changes and Applicability to Polyploids.

Notes	Designation	Exemption	Diploids	Auto-ploids	Allo-ploids	GOF
	§ 340.1(b)(1)	The genetic modification is a change resulting from cellular repair of a targeted DNA break in the absence of an externally provided repair template	1 pair of homologous chromosomes			yes
	§ 340.1(b)(2)	The genetic modification is a targeted single base pair substitution	1 pair of homologous chromosomes			yes
	§ 340.1(b)(3)	The genetic modification introduces a gene known to occur in the plant's gene pool or makes changes in a targeted sequence to correspond to a known allele of such a gene or to a known structural variation present in the gene pool.	1 pair of homologous chromosomes			yes
Overarching LOF exemption	340.1(b)(4)(vi)(A M1) on the exemptions-confirmations website	any diploid or autopolyploid plant that contains any combination	all alleles of a single genetic locus on homo-	all alleles of a single genetic locus on homo-	any combination of loss of function modification	no

		of loss of function modification -s in one to all alleles of a single genetic locus, or any allopolyploid plant with any combination or loss of function modification in one or both alleles of a single genetic locus on up to four pair of homoeologous chromosomes, without the insertion of exogenous DNA	logous chromosomes	logous chromosomes	tions in one or both alleles of a single genetic locus on up to four pairs of homoeologous chromosomes	
Deletion of any size; one or two cuts; external repair template for deletion diploids and autopolyploids	340.1(b)(4)(vi)(A M2) as listed in the exemptions- confirmations website	a single contiguous deletion of any size, resulting from cellular repair of one or two targeted DNA breaks on a single chromosome or at the same location(s) on two or more homologous chromosomes, without insertion of DNA, or	Applicable	Applicable	does not apply	yes

		with insertion of DNA in the absence of a repair template				
expand §340.1(b)(2) and (3) to auto-polyploids	340.1(b)(4)(vi)(A M3) as listed in the exemptions- confirmations website	The genetic modification is a targeted single base pair substitution or the genetic modification introduces a gene known to occur in the plant's gene pool or makes changes in a targeted sequence to correspond to a known allele of such a gene or to a known structural variation present in the gene pool	not relevant	Applicable	does not apply	yes
allow up to 4 multiplex or sequential modifications	340.1(b)(4)(vi)(A M4) as listed in the exemptions- confirmations website	Any combination of up to 4 multiplexed or sequentially made modifications provided that each edit is at a different genetic locus and would individually qualify for an existing exemption	Applicable	Applicable	Applicable	for allopolyploids, multiple heterozygous modifications are applicable

process for further modificati on of exempt plants	340.1(b)(4)(vi)(A M5) in the exemptions- confirmations website	Plants that have previously completed voluntary confirmation process and have been produced, grown, and observed consistent with conventional breeding methods for the appropriate plant species, could be further modified in accordance with the exemptions	Applic- able	Applic- able	Applic- able	for allopolyploids, multiple hetero- zygous modificat ions are applic- able
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After reviewing any comments we receive, we will announce in a future notice our decision regarding any modifications that plants can contain and qualify for exemption.

Authority: 7 U.S.C. 7701-7772 and 7781-7786; 31 U.S.C. 9701; 7 CFR 2.22, 2.80, and 371.3.

Done in Washington, DC, this 7th day of November 2023.

Michael Watson,

Acting Administrator,

Animal and Plant Health Inspection Service.